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Epigenetic discrimination of identical twins from blood under the forensic scenario

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Highlights

- Epigenetic discrimination of 10 monozygotic twin pairs was investigated under the forensic scenario.
- Genome-wide methylation array analysis in blood reference DNA identified numerous candidate markers.
- Top 3 markers were validated using methylation-specific qPCR and tested in trace DNA.
- 67.85% and 68.5% of markers showed >0.1 twin differences in reference and trace DNA, respectively.
- A proportion of twin-differentiating markers in reference DNA may be non-informative in trace DNA.

Abstract

Monozygotic (MZ) twins share the same STR profile, demonstrating a practical problem in forensic casework. DNA methylation has provided a suitable resource for MZ twin differentiation; however, studies addressing the forensic feasibility are lacking. Here, we investigated epigenetic MZ twin differentiation from blood under the forensic scenario comprising i) the discovery of candidate markers in reference-type blood DNA via genome-wide analysis, ii) the technical validation of candidate markers in reference-type blood DNA using a suitable targeted method, and iii) the analysis of the validated markers in trace-type DNA. Genome-wide methylation analysis in blood DNA from 10 MZ twin pairs resulted in 19 to 111 twin-differentially methylated sites (tDMSs) per pair with >0.3 twin-to-twin differences. Considering all top three candidate tDMSs across all pairs in the technical validation based on methylation-specific qPCR, 67.85% generated >0.1 twin-to-twin differences. Of the validated tDMSs, 68.4% showed >0.1 twin-to-

twin differences with qPCR in trace-type DNA across 8 pairs. Using an updated marker selection strategy, 8 additional candidate tDMSs were obtained for an example MZ pair, of which 7 showed >0.1 twin-to-twin differences in both reference- and trace-type DNA. Lastly, we introduce a high-resolution melting curve analysis of the entire fragment that can complement the proposed approach. Overall, our study demonstrates the general feasibility of epigenetic twin differentiation in the forensic context and highlights that the number of informative tDMSs in the final trace DNA analysis is crucial, as some candidate markers identified in reference DNA were shown not informative in the trace DNA due to various, including technical, reasons. Future studies will need to address the optimal number of epigenetic markers required for reliable identification of MZ twin individuals including statistical considerations.

Keywords

Monozygotic twins, forensic epigenetics, DNA methylation, Illumina 450K array, quantitative PCR, whole blood

1. Introduction

Individual identification of monozygotic (MZ) twins in either criminal or paternity casework has always been challenging. Since MZ twins are derived from the same zygote, they share a literally identical genomic DNA sequence. Except for very rare cases [1], conventional DNA profiling techniques using short tandem repeat (STR) genetic markers typically fail to distinguish between individuals within the same MZ twin pair. This inevitably leads to a dead-end in police investigation and may result in court decisions of prime suspects being set free, which highlights the need for a suitable approach to be applied in such cases.

There is increasing evidence that MZ twins can demonstrate a very small degree of genetic differences, in terms of single nucleotide polymorphisms (SNPs) [2], copy number variations (CNVs) [3-5], Y-chromosome satellite DNA [6], and bone-marrow-derived memory B lymphocytes DNA sequences [7]. However, these sequence differences are extremely rare and difficult to locate. Furthermore, their detection in forensic-type samples might be impractical in case that they are seen only in a small portion of cells (mosaicism). Proposed genome-wide screening methods, such as ultra-deep whole genome sequencing [2], require DNA of high quality and quantity and lead to high costs.

Contrary to the stable genomic DNA sequences, epigenetic patterns, especially DNA methylation, are more dynamic as they are influenced by genetic, environmental and stochastic factors, the latter two varying throughout an individual's life [8, 9]. DNA methylation differences between both phenotypically concordant and discordant MZ twins have been studied previously, not only to investigate naturally occurring variation in DNA methylation and epigenetic heritability [10-14], but also to unravel the impact of epigenetics in development and ageing [15, 16] as well as disease [17-20]. The observed epigenetic drift within MZ twin pairs can be seen at

a genome-wide level [12] and also at specific loci [21, 22], which are most likely linked with gene regulation involved in specific phenotypes.

Although the extent of these differences, and to what degree they are shared by different MZ twin pairs, is yet not understood, the potential of using DNA methylation to distinguish between MZ twins for forensic purposes has already been recognized [23-28], albeit via limited studies. In an initial study, Li *et al*. tested blood DNA samples of 22 adult MZ twin pairs by using the Illumina Infinium Human Methylation 27K BeadChip array that allows for the co-analysis of 27,578 CpG sites, which revealed significant DNA methylation differences (377 CpG sites, methylation difference >0.17) [23]. Applying the criterion of how frequent the observed methylation differences were amongst MZ twin pairs, authors sub-selected a total of 92 CpGs, that were differentially methylated in all 22 pairs [24]. Furthermore, in another candidate-finding approach, Du *et al* [25] analysed four MZ twin pairs with methylated DNA immunoprecipitation (MeDIP) and identified a set of 38 differential methylation regions demonstrating pair-specific methylation differences, all of which are located within CpG islands (<500bp long, >55% GC content, [29]). The majority of these markers are associated with cell differentiation, proliferation and development, but future validation is necessary to reveal whether they can be used for MZ twin differentiation in routine forensic practice.

Using a more targeted approach, forensic researchers have also explored the possibility of using differential methylation patterns within satellite DNA, and more specifically within interspersed repeats such as LINE-1 [26] and Alu sequences [27]. LINE-1 methylation was investigated in both blood and buccal cells from a total of 119 MZ pairs using bisulfite pyrosequencing. Authors detected statistically significant tissue differences and were able to distinguish only a subset (12.61%) of MZ pairs (n=15 pairs) using this specific region (3 CpG

sites) in blood [26]. Also, the detected CpG methylation was significantly correlated with gender in blood and with age in buccal cells (p=0.001), highlighting the complexity of epigenetic patterns. Lastly, in the study by Stewart *et al* [27], all 5 MZ twins tested were distinguished by analysing Alu methylation in buccal cells (2 fragments, 19 CpG sites). The authors used high-resolution melting curve analysis, which can differentiate between fragments having differential methylation levels on the basis of different melting temperatures. Limitations of such approach include the low resolution (investigation of an entire fragment rather than specific CpG sites), the required large sample volume and the questioned applicability in other tissues (such as blood or saliva) [27].

In the present study, our aim was to execute epigenetic differentiation of MZ twins under the forensic scenario, comprising i) the discovery of candidate markers in reference DNA via genome-wide screening, ii) validating selected candidate markers in reference DNA using a method suitable for forensic trace analysis, and iii) applying such suitable method for analysing the validated markers in trace DNA. To the best of our knowledge, this is the first study addressing the differentiation of MZ twins in reference-type samples as well as in forensic-type samples, using the combination of genome-wide screening and methylation-specific qPCR for targeted analysis.

2. Materials and Methods

2.1. Sample collection

This study was approved by the St Thomas' Hospital Local Ethics Committee, and all participants provided signed informed consent prior to sample collection. In total, 10 pairs of female MZ twins from the TwinsUK cohort [30], aged 52-62 years and of European ancestry were included in the study. Whole blood was collected in EDTA-treated tubes as reference sample, while a small amount of blood (a small drop) was also used to make up small bloodstains on cotton material

(~0.4-0.5cm^2 stains when dried). Samples were stored at -80^{o}C before proceeding to DNA extraction and analysis. The monozygosity of the twins used in the study was confirmed by genotyping 15 highly polymorphic STR loci using the AmpFLSTR™ Identifiler™ PCR Amplification kit (Thermo Fisher Scientific, USA), which resulted in the same STR profile per MZ pair respectively.

2.2. DNA sample preparation

Total DNA from whole blood, representing reference-type DNA samples, was extracted using the DNeasy Blood & Tissue kit (QIAGEN, Germany) according to the manufacturer's instructions. Genomic DNA from the dried bloodstains on cotton, representing trace-type DNA samples, was isolated using the QIAamp® DNA Investigator kit (QIAGEN) following the recommended protocol for 'Isolation of total DNA from body fluid stains'. The whole blood stain was used for DNA extraction and DNA samples were eluted in 20µl. To maximise DNA yield, samples were lysed at 56^{o}C for at least 2 hours and QIAshredder spin columns (QIAGEN) were used to harvest lysate remaining in the cotton material. To access the accuracy and linearity of methylation quantification, 7 DNA standards of known methylation levels (0, 0.1, 0.25, 0.5, 0.75, 0.9, 1) (EpigenDx, USA) were also analysed. All DNA samples were quantified using the Quantifiler® Human DNA Quantification kit (Applied Biosystems, USA), according to the manufacturer's instructions. For genome-wide analysis, 750ng of extracted DNA was bisulfite-converted using the EZ DNA Methylation™ Kit (ZymoResearch, USA). For qPCR analysis, depending on the available DNA quality and/or quantity, 200ng (whole blood), 20ng (bloodstains) and 250ng (DNA standards) of DNA were treated using the MethylEdge™ Bisulfite Conversion system (Promega, USA). Considering 80% DNA recovery after conversion as suggested by the manufacturer, bisulfite DNA samples were diluted down to 1ng/µl.

2.3. Genome-wide DNA methylation profiling

Genome-wide DNA methylation profiles used in this study were generated using the Illumina Infinium Human Methylation 450K BeadChip array (Illumina, USA). Intensity images were captured by GenomeStudio (2010.3) Methylation module (1.8.5) software. The Illumina 450K BeadChip assay allows for quantification of DNA methylation levels at 485,512 CpG dinucleotides. For each CpG site, a beta value was estimated, ranging from 0, representing completely unmethylated, to 1, representing completely methylated sites. The beta value is interpreted as average methylation in a particular site taking into account the collection of cells that form each sample. The Human Methylation 450K BeadChip array contains two types of beads associated with the two different chemical assays, Infinium I and II, which can potentially cause bias in probe design [31]. DNA methylation probes that mapped to multiple locations to the reference sequence (with exact sequence match and within up to two base pair mismatches) and probes that contain a non-rare polymorphism in the CG site, minor allele frequency (MAF)>0.1 in European population from 1,000 genomes, were removed. We only considered autosomal probes in the analysis. In total, we restricted our downstream analyses to 438,756 probes. Due to the limited and subtle DNA methylation differences expected within each MZ pair (unlike a healthy *versus* diseased tissue), we applied appropriate normalisation approaches to reduce technical variability and aiming to improve statistical power. We performed two types of normalisation of the 450K array results, subset-quantile normalisation (SWAN) [32] and functional normalisation (FUNNORM) [33], both implemented in Bioconductor package Minfi [34]. SWAN performs within-array and between-array normalisation to correct for possible technical biases introduced by the use of two assay types (Infinium I and II) in the array with different chemistries. In addition, functional normalisation is intended to eliminate other non-biological variation known as batch

effects. In the normalised datasets, the correlation between MZ twins for each MZ twin pair was calculated using Pearson's correlation across all CpG sites.

2.4. Selection of candidate tDMSs

Genome-wide data normalised by both the FUNNORM and SWAN methods were used to select potential tDMSs for each MZ twin pair. Firstly, the absolute methylation difference for each CpG site within each MZ twin pair was calculated in both datasets. FUNNORM methylation differences were categorised by applying different thresholds of absolute differences between MZ twins: 0.05, 0.10 and 0.3. Only the CpG sites demonstrating >0.3 difference were considered for further analysis; this threshold was chosen to account for the microarray and processing methods' potential capacity to accurately and reproducibly quantify DNA methylation levels. Only candidate tDMSs also confirmed in the SWAN approach by sharing the same level of methylation difference (>0.3) were then considered further. For each pair the 3 tDMSs that showed the largest differences were chosen for validation [Table S1]. Moreover, for one MZ twin pair, an extra 12 tDMSs were also selected for validation. Due to an overlap in CpG-sites identified as tDMSs in more than one MZ pair, a total of 34 tDMSs were selected for validation.

2.5. Design of methylation-specific PCR approach

Selected array-derived candidate tDMSs were validated in the same reference DNA used for array analysis by means of qPCR. The developed method is a SYBR green-based PCR assay for the dual analysis of DNA methylation and CpG methylation density of the entire fragment. The protocol is based on previous studies [35, 36] but has been adjusted for more targeted CpG-specific analysis suitable for the analysis of forensic-type samples, typically of low DNA quantity and/or quality.

The method uses two rounds of PCR amplification; firstly, an end-point, bisulfite-specific PCR amplifying the genomic region containing the CpG site of interest, followed by two qPCR reactions, one targeting the specific methylated site using methylation-specific primers and a second nested one using methylation-independent primers [Figure 1]. The first step of the end-point bisulfite-specific PCR also has the potential to be multiplexed to enable the simultaneous amplification of many target CpG sites and therefore, to increase sensitivity, which can be essential when analysing forensic casework samples. Moreover, the use of SYBR green dye allows for introducing a melting curve analysis step of the final target amplicons and the determination of the methylation density of all CpG sites included in the target sequence. The same qPCR approach was used for blood trace DNA methylation profiling. A flow diagram of the entire process with the amounts of sample to be used in each step can be found in Figure S1.

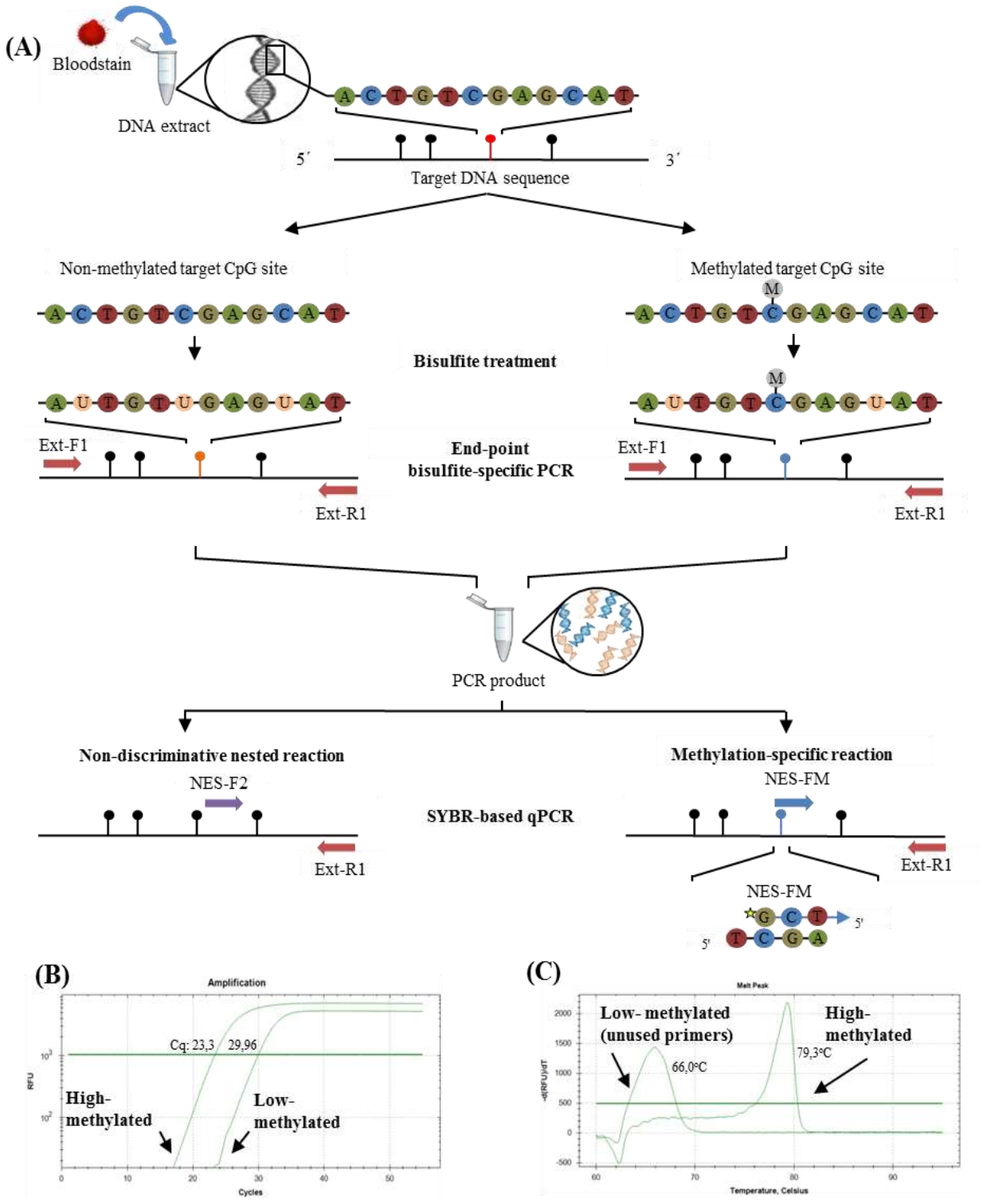


Figure 1. Methodological approach used for tDMS validation and final trace analysis
(A) Schematic representation of the developed method from DNA extraction to methylation-specific qPCR, (B) amplification curves (log(RFU) vs. PCR cycles) and (C) melting peak analysis (-d(RFU)/dT vs. temperature) of both low- and highly-methylated DNA standards using a methylation-specific primer.

The chromosomal location of the selected tDMSs was confirmed using the online Ensemble genome browser (human GRCh37/hg19 genome). Primers were designed using the online-tool BiSearch [37], specifically intended for methylation analysis. As explained above, for each assay four primers were designed, including an external forward primer (EXT-F1), an external reverse primer (EXT-R1) and, depending on the assay, a nested forward or reverse primer (NES-F2 or NES-R2) as well as a nested methylation-specific reverse or forward primer (NES-RM or NES-FM). Where possible, the external primers were designed so that they contain no CpG site to ensure methylation-independent amplification of the DNA template. To ensure similar amplification efficiencies between the two qPCR reactions (methylation-independent and methylation-specific), which further facilitates the quantification analysis, only one nested non-discriminatory primer was used, which was designed so it is 'shifted' by as few bps as possible (2-3bp) from the position of the corresponding methylation-specific primer. Furthermore, it has been previously proposed that the presence of a locked nucleic acid (LNA) at the 3'-most end of the methylation-specific primer enhances primer binding and allows for efficient discrimination between the sequences corresponding to the two methylation states [38]; therefore, LNA-containing methylation-specific primers were used in this study. The designed assays together with the primer sequences can be found in Supplementary Table S2.

2.6. End-point PCR amplification of bisulfite-treated DNA

During the first end-point PCR step, 1ng of bisulfite-treated DNA was amplified using the designed bisulfite-specific primers [Table S2]. For each PCR assay, the primer annealing temperature, primer and $MgCl_2$ concentration were optimised; however, one out of 34 PCR assays (cg09738481) failed to pass this optimisation step resulting in no or multiple PCR signal and was,

therefore, excluded from further analysis. All remaining assays were amplified either using the ZymoTaq™ Premix (ZymoResearch) or the QIAGEN Multiplex PCR kit (QIAGEN), as specified in Table S2. For each reaction, 6.5µl of 2X ZymoTaq Premix or 2X QIAGEN Multiplex PCR Master mix, 1µl of 4X PCR primer mix (final concentration of 0.3µM for each primer), 1µl of 25mM $MgCl_2$ (only for the ZymoTaq™ reactions for a final concentration of 3.67mM as the buffer already contains 1.75 mM $MgCl_2$), 1µl of bisulfite DNA template (1ng) and 3.5µl (or 4.5µl for the QIAGEN reactions) of nuclease-free water, for a total reaction volume of 13µl. The thermocycling program used was: 95°C for 10 (ZymoTaq™) or 15 (QIAGEN) minutes, followed by 32 cycles of 94°C for 30 seconds, T_a for 35 seconds, 72°C for 35 seconds, and a final extension step of 72°C for 7 minutes. T_a is specified in Table S2. Following amplification, the amplicon quality was assessed on a 2% agarose gel. Depending on the PCR efficiency, PCR products were diluted 400-4200 times [Table S2]. All samples including DNA standards, reference samples and bloodstains were analysed in duplicate.

2.7. Quantitative PCR (qPCR) assay coupled with melting curve analysis

During the second real-time qPCR step, 1µl of diluted PCR product was amplified using the designed methylation-independent and -specific primers [Table S2]. For each methylation-specific qPCR, the primer annealing temperature was also optimised using a temperature gradient. Five qPCR assays (cg23066587, cg08299859, cg14754187, cg24719020 and cg26695881) failed to pass this second optimisation step resulting in very low C_T (PCR cycle number required to reach the threshold) and were, therefore, excluded from further analysis. All qPCR reactions were performed using SsoAdvanced™ Universal SYBR® Green Supermix (BIO-RAD, USA) on a CFX384 Touch™ Real-Time PCR Detection system (BIO-RAD). For each reaction, 5µl of 2X Sso

Advanced universal SYBR® Green supermix, 1µl of 2X PCR primer mix (final concentration of 0.2µM for each primer), 1µl of diluted PCR product and 3µl of nuclease-free water, for total 10µl. The thermocycling program used was: 98ºC for 3 minutes, followed by 40 cycles of 95ºC for 15 seconds, T_a for 35 seconds and 60ºC for 35 seconds. T_a is specified in Table S2. Following amplification, a melt-curve analysis was performed by introducing one cycle of dissociation step from 65ºC to 95ºC (0.5 ºC increment, 2 sec/step). All samples were analysed in duplicate, corresponding to a total of four replicates for each sample.

2.8. qPCR data analysis

The difference of C_T between the two qPCR reactions (ΔC_T) was calculated for each sample. As a first quality control step, samples that showed a ΔC_T outside the range 'mean ± standard deviation ΔC_T' for each assay were excluded from analysis. This is to account for random, sub-optimal amplification. Raw fluorescent values obtained by qPCR were then analysed using the LinRegPCR software [39]. The principle of LinRegPCR software is based on a sample-independent analysis that starts with a baseline correction, followed by the calculation of a window-of-linearity and both the individual PCR efficiency per sample and mean PCR efficiency per amplicon. A baseline fluorescence correction per sample was first performed by plotting the Log(fluorescence) versus the cycle number in a linear regression approach. An accurate baseline estimation is key for the correct estimation of individual PCR efficiencies. When the baseline-to-plateau distance is too short, the baseline fluorescence correction for a sample can fail, indicating that the qPCR conditions should be optimised. Furthermore, the built-in iterative algorithm then detects the range of PCR cycles that the sample undergoes linear amplification (highest R^2 value) during the exponential phase and uses this subset of data points to calculate the so-called Window-of-

Linearity. The individual PCR efficiency per sample in this linear regression approach is defined as fold increase per cycle and is calculated as Eff = 10^{slope}. PCR efficiencies are given values ranging from 1 to 2, where 2 represents a perfect doubling of the amplicon's concentration in each cycle. Samples with PCR efficiency values lower than 1.5 were excluded from analysis. This type of analysis does not use the assumption that PCR efficiency is the same in all samples, but accounts for potential effects of individual PCR reaction parameters such as PCR inhibitors. This observation was confirmed in this study since many different amplicons and primer sets were analysed. This analysis leads to a value of starting DNA material per sample (N0) using the equation 'baseline÷(Eff^C_T)', expressed in arbitrary fluorescence units. N0 values obtained by the methylation-specific qPCR reactions were normalised against the methylation-independent qPCR reactions to account for differences in PCR efficiency, initial PCR product template applied in the qPCR and pipetting errors. N0 values obtained by the DNA methylation standards were used to create a calibration curve per assay. Methylation ratios of MZ twin DNA samples were subsequently calculated using the resulting equations [an example can be found in Supplementary Figure S2]. Lastly, the average temperature of melting peaks of all qPCR replicates was calculated.

3. Results & Discussion

3.1. Genome-wide DNA methylation patterns of MZ twin reference DNA

We first explored the correlation between MZ twins in blood DNA considering all CpG sites in the final genome-wide microarray dataset considered for downstream analysis. While the employed Illumina 450K platform has previously shown high reproducibility [24, 40], beta values (methylation ratio) underwent two different normalisation approaches to assess technical bias. Using functional normalisation, the average correlation across all CpG sites within a MZ twin-

pair, considering all MZ twin pairs, was 0.9967 (range 0.996 to 0.998). Similar estimates were observed with an alternative normalization approach SWAN (data not shown). An example genome-wide DNA methylation profile for one MZ twin pair is shown in Figure 2A.The observed high correlation between MZ twins within pairs is in line with previous genome-wide estimate from MZ twins [24, 28]. The small DNA methylation differences between MZ twins within pairs may be attributed in part to stochastic processes and MZ discordance for environmental exposures, phenotypes, and diseases leading to epigenetic footprints. The focus of the present study was to explore the value of these epigenetic differences to differentiate MZ twins within pairs.

3.2. Identification of candidate tDMSs in reference-type DNA

To identify candidate tDMSs per MZ twin pair, we calculated the within-pair absolute difference in DNA methylation levels for all 438,756 CpG sites tested on the Illumina 450K array, and assessed the number of tDMSs with blood methylation differences larger than 0.05, 0.10 and 0.3, respectively (beta values) [Figure 3A]. The results were similar across both FUNNORM and SWAN normalisation approaches. Considering CpG sites that surpassed the difference threshold in both normalisation methodologies, and averaging over 10 tested MZ twin pairs, the following proportions of tDMSs were observed (average ± standard deviation): 7.94% (34,840 ± 8,413), 1.27% (5,586 ± 3,230) and 0.013% (56 ± 32), for a methylation level difference of >0.05, >0.10 and >0.3, respectively [example in Figure 2B]. These findings suggest that tDMSs within MZ twin pairs are subtle, yet substantial. Furthermore, the distribution of the detected large differences (>0.3) is shown in Figure 3B; depending on the twin pair there are 1 (pair 1) to 22 (pair 7) candidate tDMSs at 0.7 threshold of twin-to-twin methylation difference.

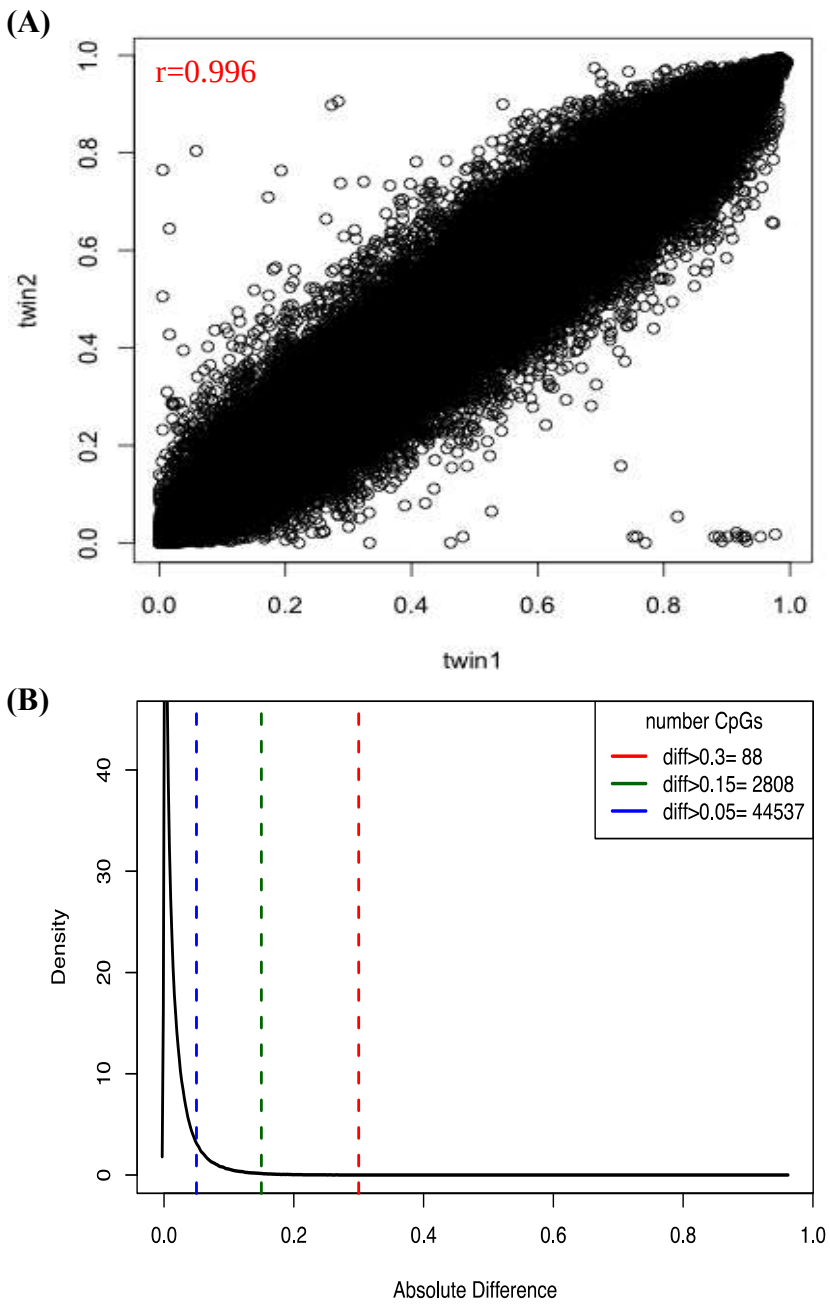


Figure 2. Genome-wide DNA methylation and tDMS analysis in an example MZ twin pair
(A) Genome-wide DNA methylation profiles from microarray analysis, and (B) selected tDMSs using FUNNORM normalisation.

(A)

MZ twin pair	No of array-derived candidate tDMSs at each methylation cut-off point								
	FUNNORM			SWAN			Common		
	0.05	0.10	0.3	0.05	0.10	0.3	0.05	0.10	0.3
1	26890	2925	29	29945	2921	60	20076	1656	19
2	49269	12762	92	52528	12043	65	43020	12041	36
3	52212	10949	54	51455	9117	50	44913	7646	30
4	44537	10550	88	47775	9710	79	39184	8100	59
5	51421	7221	95	46517	4646	80	39607	3602	53
6	38484	5316	51	36074	3959	71	30202	2980	40
7	32958	2897	143	40137	2897	184	23385	4758	111
8	42265	7608	106	39575	5715	133	41548	4231	84
9	40942	5400	62	37372	3963	71	31486	2881	32
10	45274	11662	182	48666	10659	132	34975	7965	99

(B)

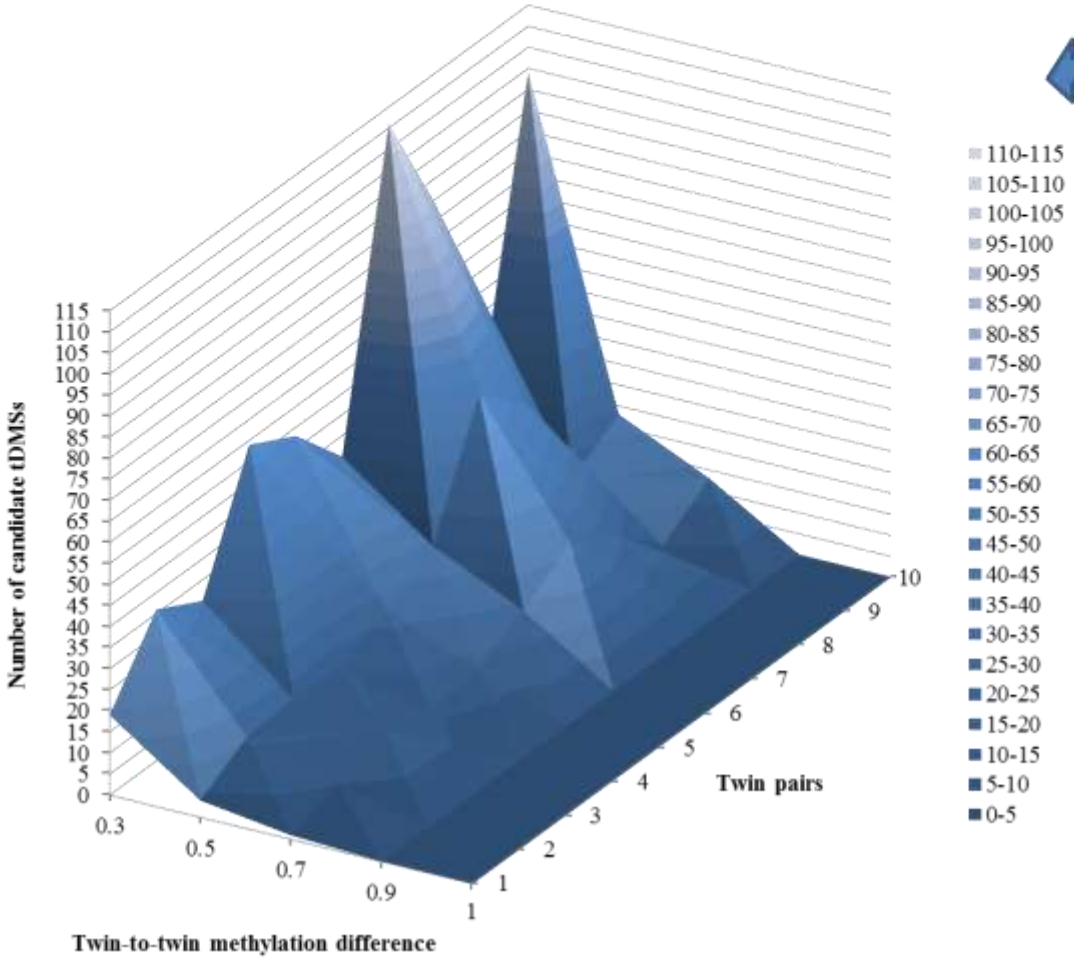


Figure 3. Array-derived candidate tDMSs in reference DNA of 10 MZ twin pairs
(A) Number of markers in both normalisation methods at different methylation difference cutoffs, (B) 3D surface plot showing the distribution of the number of tDMSs demonstrating large differences (>0.3) by both normalisation approaches.

For further analysis, we focused on the common tDMSs that overlap between both normalisation methods using the methylation difference threshold of 0.3. As a result, the number of array-derived candidate tDMSs ranged between 19 and 111 depending on the investigated MZ twin pair [Figure S3A]. The majority of these differences are pair-specific, although there are some tDMSs that are shared among twin pairs. At the 0.3 cutoff and considering all twin pairs, we detected a total of 433 tDMSs, from which 358 were unique and pair-specific, while the remaining 75 (17.31%) are shared among two to five MZ pairs. Particularly, 9% are shared among two pairs, while 4.39%, 3.46% and 0.46% are shared among three, four and five pairs, respectively.

Moreover, the majority of these tDMSs (68%), found equally on both the forward and reverse DNA strands, are located in or near genes [Figure S3B], with approximately half located in the gene body (47%) and a proportion in other functional regions such as the 5' untranslated region (5'-UTR) (7%) [Figure S3C]. Furthermore, according to Illumina, 19% of the identified tDMSs at the 0.3 threshold are located in enhancers, indicating their potential role in regulating gene expression. Lastly, the probes used in this array have been designed to specifically target CpG islands (CGI) (<500bp, GC content>55%, [29]), CGI shores (up to 2kb from CGI), CGI shelves (between 2-4kb from CGI) and non-CpG island regions (>4kb from CGI). We investigated whether tDMSs appear more often in specific chromosomal regions according to CpG content, and observed that 27% of tDMSs are annotated to fall in CGIs, 25% in CGI shores, 6% in CGI shelves and 42% are non-CGI probes [Figure S3D]. At this cutoff, it seems that CGI probes are highly represented, which has been previously supported by similar studies [25, 28].

3.3. Technical validation of array-derived candidate tDMSs in reference-type DNA

As described in the methods, for each MZ twin pair the top 3 tDMSs identified by both functional and subset-quantile normalisation were selected for technical validation [Table S1] by employing CpG-specific qPCR approach in the same reference-type DNA samples previously used for microarray analysis. Since some of these top tDMSs (n=6, cg25949955, cg08240074, cg05905030, cg09007841, cg01585094, cg10617763) overlapped across MZ twin pairs (up to 3 pairs), the final set of candidate tDMSs involved in the validation testing comprised of 22 CpGs in total. The majority of selected tDMSs (12 out of 22) were located in genes, specifically *ISM1*, *SLC12A7*, *NXN*, *MYH7*, *RPTOR*, *GPR125*, *PLK1*, *SLIT1*, *ANGPT1*, *MLF2*, *RNF44* and *CPNE7*. The associated genes have been previously reported to be involved in biological processes such as cell growth and differentiation, metabolism, insulin recycling, vascular and neural development, and have also been associated with common health conditions like cancer, tumor angiogenesis, cardiomyopathy and myeloid leukemia. The normalised methylation levels at these top tDMSs as detected in the array are presented in Table S3. In all cases, the expected methylation differences within a MZ twin pair were substantial, as these candidate CpGs seem to be either un-methylated or fully methylated as suggested by the array data.

The importance of technical validation of the methylation results obtained from genome-wide DNA methylation screening approaches, such as microarray analysis used here, by using more quantitative, locus-specific assays has been highlighted before [41], but has not yet been adopted in most of the previous forensically motivated studies addressing MZ twin differentiation. Validating the candidate tDMSs is needed not only to assess the quality and accuracy of the obtained data, but also to determine whether the specific detected differences observed between MZ twins in the array analysis are reproducible across technologies. This is especially important

in forensic epigenetic applications where screening methods to identify candidate markers such as microarrays are not suitable for trace DNA analysis due to DNA quality and quantity restrictions in forensic material. Despite the use of two normalisation methods on the microarray data, technical validation in the same DNA used for microarray analysis is still required to assess reproducibility of the findings. For this purpose, and for subsequent trace DNA analysis, we developed a CpG-specific qPCR approach, as outlined in the methods section. DNA methylation data were derived by applying a standard curve from known DNA methylation standards for each assay; an example of this analysis can be found in Figure S2. Overall, the DNA methylation results obtained from this technical validation step in the same reference-type DNA samples used for array analysis are presented in Table 1.

Taking into account all candidate tDMSs, using our qPCR approach we successfully detected at least 0.1 twin-to-twin difference for 19 tDMSs (67.85%), while 9 tDMSs (32.15%) gave lower (<0.1) methylation differences. As shown in Table 1, using the qPCR approach in MZ twin's reference DNA samples did not generally show non-methylation/fully methylation profiles as previously observed in the array analysis, but there were some exceptions, specifically the top 3rd tDMS in twin pair 3 (cg04359702) with 0.705 qPCR difference and top 2nd tDMS in twin pair 5 (cg27326062) with 0.715 qPCR methylation difference. There were also some cases where one twin showed a very low or high methylation profile as expected from array data, while the other twin demonstrated intermediate methylation, not expected from array data. For example, for top 1st tDMS in twin pair 4 (cg26886231), twin 4A had no methylation while twin 4B had a value of 0.586. Nevertheless, and most importantly, the methylation differences kept the same direction across the two methods for 22 out of the 28 tDMSs, meaning twin A showing higher methylation than twin B, or vice versa.

Our difficulty to qPCR-replicate the exact array-based methylation differences at most of the tDMSs could be partly explained by potential technical batch effects in the Illumina 450K array, such as potential probe bias, potential variability in M *vs.* U bead intensity signal differences, albeit our normalization approach tried to minimise those, and/or variation introduced by incomplete bisulfite conversion. DNA methylation detection inaccuracies due to probe bias have also been mentioned before [31] and could be explained either by the probe mapping to multiple genomic locations (which we removed in our study) or by a probe sequence harboring a SNP site commonly present within a sample and hence, influencing its ability to hybridise DNA. Nevertheless, studies on the impact of SNPs on the probe sequences show that, unless the SNP is

on the target CpG site, they do not significantly affect hybridization [42]. On the other hand, many probes contain additional CpGs, which may introduce another base pair mismatch depending on their methylation status, hence affect probe binding and methylation detection (especially regarding Infinium design type I probes). For example, at cg01585094 we observed discrepancies for both twin pair 10 (top 3rd tDMS) and twin pair 7 (top 2nd tDMS), which could be explained by the presence of four additional CpGs on the probe sequence. A discrepancy was also observed for top 1st tDMS in twin pair 8 (cg00922825), where both twins showed a very low methylation profile with the qPCR method (0.19). Here, an explanation could lay behind the fact that this particular site for twin 8B had not been converted during bisulfite treatment in the genome-wide experiment (hence 0.97 methylation) [Table S3], resulting in a false-positive tDMS identification.

Lastly, we cannot exclude inter-methodological differences between the probe-based array experiment and the PCR-based targeted approach, as each uses a different chemistry. For example, potential occurring amplification bias together with possible errors during PCR product dilution in our targeted experiments cannot be excluded, highlighting the need to assess the performance of the developed qPCR assays before moving to the forensic-type samples. For extra validation, we also sequenced our PCR products with a standard Sanger sequencing protocol, which resulted in similar DNA methylation values with the ones obtained by qPCR for all tested tDMSs (data not shown). We understand that we do place a lot of trust on quantifying exact methylation values between the methods, which might not be possible in the end (except for perhaps in the case of ultra-deep bisulfite sequencing, but even then there could be issues with the surrounding sequence). Looking at the raw methylation data across microarray vs qPCR values [Tables 1 and S3], we observed ‘same’ methylation levels in only ~25%. Hence, while from a forensic standpoint we would like to have methylation values and differences as precise as possible when comparing

these datasets obtained by two methods, we might need to re-evaluate our approach of calculating validation success rates in future studies.

3.4. Analysis of validated tDMSs in trace-type DNA

Despite the observed discrepancies, we detected substantial differences between twin individuals within each MZ twin pair also with our qPCR approach. For twin pairs 1, 4, 5, 6 and 9, substantial methylation twin-to-twin differences at the 0.1 level were qPCR-verified for all array-derived candidate tDMSs tested [Table 1], while for pair 7 only one candidate tDMS was validated at the 0.1 level, and for pairs 8 and 10, we were unable to verify at the 0.1 level any of the candidate tDMSs tested. Moving to the next step in the forensic scenario of epigenetic MZ twin differentiation, we qPCR-tested the technically validated tDMAs with >0.1 methylation differences (19 out of 28 tDMSs, in 8 out of 10 MZ twin pairs) in the trace-type DNA derived from small blood stains produced from the same blood of which the reference-type DNA were obtained [Figure 4]. Taking into account all replicates analysed from the reference DNA, we estimated the standard error of each CpG assay, which ranged between 0.012 and 0.102, suggesting that the used qPCR method was both reproducible and sufficiently accurate to confidently detect 10% methylation differences. These data serve as prerequisite to use tDMSs with at least 0.1 methylation differences in the qPCR analysis of the trace DNA. Looking at all 38 tDMS comparisons [Figure 4] for 13 (34.2%) trace DNA methylation was within the expected range of standard error taking into account observed technical variation (average deviation from reference samples 0.014), 15 (39.5%) showed a considerably lower methylation in trace DNA (average deviation of -0.243) while the remaining 10 (26.3%) showed higher methylation (average deviation of 0.321) compared to the reference DNA qPCR results.

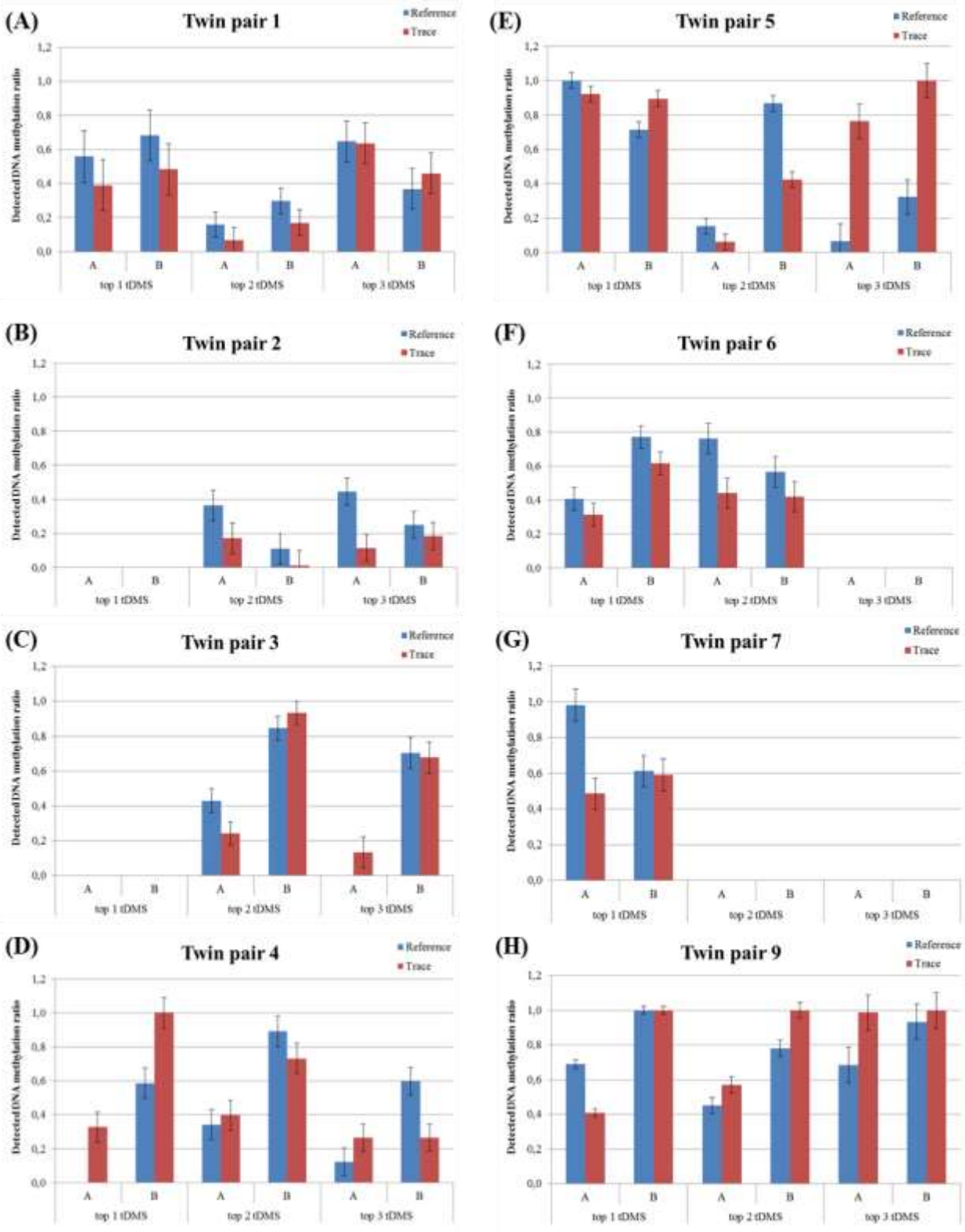


Figure 4. qPCR-based methylation levels of validated tDMSs in both reference-type and trace-type DNA for the 8 MZ pairs for which any of the three top candidate tDMS tested was validated at the 0.1 level of methylation difference in the reference DNA

We cannot exclude that different sample preparation in bloodstains due to limited availability (lower DNA amounts treated in bisulfite conversion) may not have introduced possible differences across reference and trace DNA. In some cases, such as for the top 3rd tDMS in twin pair 5 (cg05905030) [Figure 4E] the difference can be quite extreme (0.07 in reference A and 0.76 in stain A, while 0.32 in reference B and fully methylated -1- in stain B). Some of the discrepancies between reference and trace-type DNA of the same individuals are likely explained by bisulfite conversion issues, as this treatment procedure is known to be sensitive to the starting quantity of DNA and, according to the manufacturer, does not work optimally with starting amounts of <200ng. While for the reference DNA 200ng of DNA was treated with bisulfite (within the optimal DNA input range of the kit), for the trace DNA 20ng of DNA was used due to the low quantity of blood DNA available from the traces. The less efficient the bisulfite conversion is, the higher the observed methylation values can be due to 'false positive' methylation. Furthermore, due to the limited starting DNA availability in bloodstains and as seen previously in other studies [43], we cannot exclude potential amplification bias towards the unmethylated allele during the first end-point PCR, which could partly explain the detected lower methylation in trace DNA. Also, we cannot exclude that due to the minute stain the overall cell composition could be slightly different compared to the whole blood liquid sample contributing towards a very small part of the observed discrepancies. While 20ng of DNA could be considered adequate for good sampling (corresponding to ~3,000 cells), cell type-specific DNA methylation patterns in whole blood have been observed [44, 45].

Out of the 19 twin-to-twin comparisons, 13 tDMSs (68.4%) still resulted in a twin-to-twin difference of >0.1, while the remaining 6 tDMSs (31.6%) resulted in low (<0.1) methylation differences. On average, the deviation between the detected DNA methylation differences in

reference and stain sets was 0.139, which can be partly explained by the expected technical variation. However, there were certain tDMSs that failed to show significant DNA methylation difference in trace DNA, given the expectations from the validated reference DNA. For example, top 1st tDMS in twin pair 5 (cg13291296) [Figure 4E] and top 3rd tDMS in twin pair 9 (cg10617763) [Figure 4H] resulted in no difference in the trace DNA of twin A and B, respectively. This makes the selected tDMSs non-informative for MZ twin differentiation in the final trace analysis. More importantly, however, for top 3rd tDMS in twin pair 2 (cg08240074) [Figure 4B] and top 1st tDMS in twin pair 7 (cg09007841) [Figure 4G] methylation differences in the trace DNA were reversed relative to reference DNA. Such scenario would, therefore, lead to false positive identification in a practical application when only considering such markers.

3.5. Analysis of additional tDMSs in one twin pair

From our observation that some validated tDMSs showed discrepant methylation results in trace DNA compared to reference DNA leading to reduced marker sets available for MZ twin differentiation from trace material - particularly evident in the case of twin pair 7 of this study - we investigated an increased number of candidate tDMSs for this pair. For this purpose, we selected 8 additional twin pair-specific candidate tDMSs from the array data that met the cut-off criterion of >0.3 twin-to-twin DNA methylation difference. In an attempt to improve our marker selection strategy, these markers did not necessarily demonstrate a binary methylation profile (0.35-0.74 methylation difference) in the array data, which could potentially be caused by complete analysis failure in one of the two twins within a pair, for instance due to incomplete bisulfite conversion prior to array analysis. In the qPCR validation analysis using the same reference DNA as used for array analysis, 7 out of 8 tDMSs (87.5%) showed >0.1 DNA

methylation difference (ranging from 0.164 to 0.950), and were consequently analysed in the trace DNA [Figure 5]. Overall, the concordance between reference and bloodstain methylation within each twin individual was high (average deviation of 0.08, with cg27370028 in twin 7A the only exception as the bloodstain resulted in a higher methylation, 0.304). Detected methylation differences between reference-type and trace-type DNA were also concordant for most tDMSs (except for cg27370028) [Figure 5]. As partly seen and explained before, trace DNA showed generally lower methylation levels than reference-type DNA also concerning these markers. Although more data are needed to underline this conclusion, it seems that selecting candidate tDMSs showing intermediate methylation differences between twins within a pair from the array data might be as informative as the ones showing maximal differences. We also observed that this selection approach avoiding only markers with on/off methylation in the array data could actually increase the chance of avoiding markers that turn out to be non-informative in the final trace analysis, which should be considered and further explored in future studies.

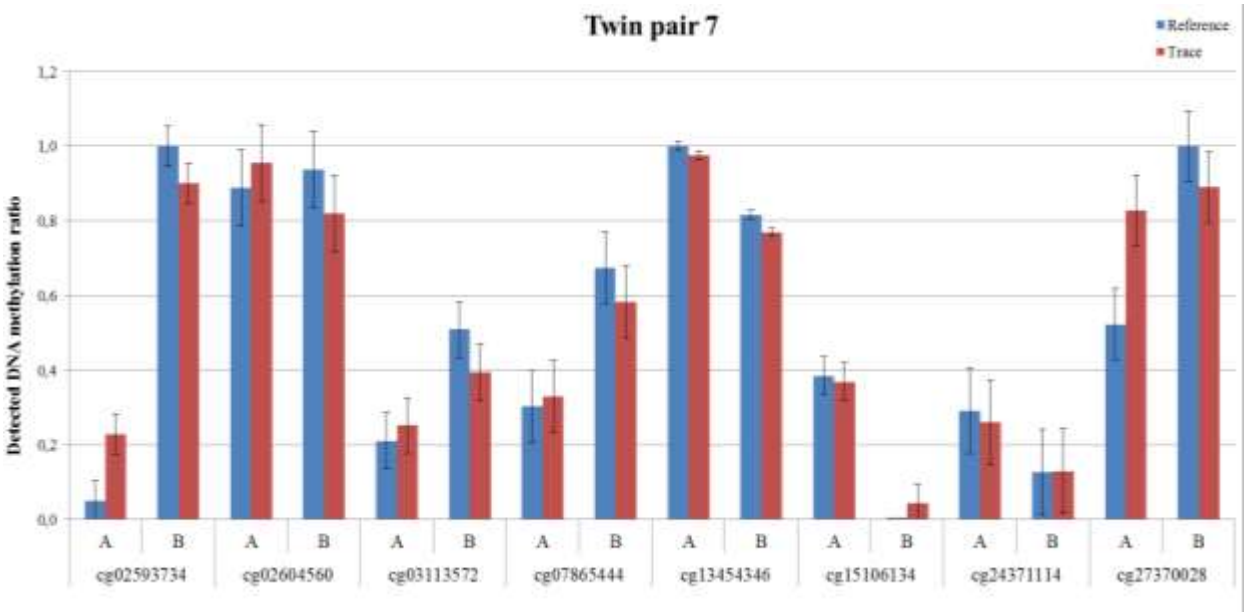


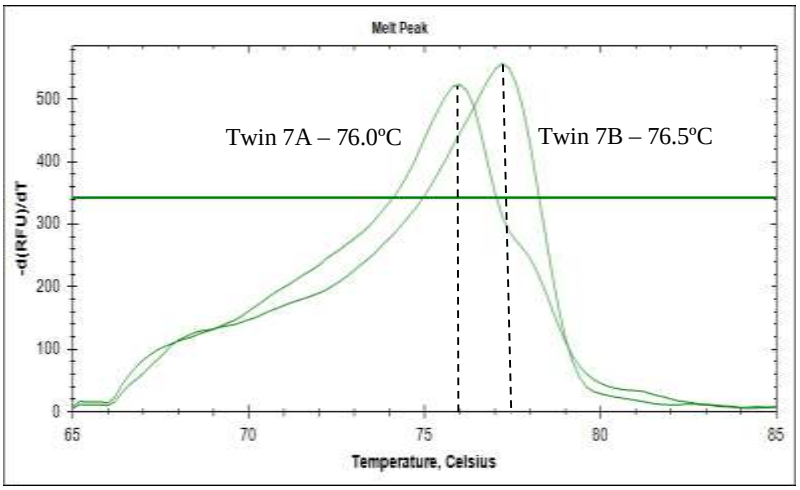
Figure 5. qPCR results of additional array-derived validated tDMSs in MZ twin pair 7 in both reference and trace DNA following an updated candidate marker selection strategy

3.6. Complementary high-resolution melting curve analysis

High-resolution melting curve analysis enables for the separation of fragments with different sequences due to their difference in CG-content and melting temperature. In the present study, we aimed to combine our proposed quantitative CpG-specific qPCR with a qualitative fragment-specific melting analysis, so that we can assess the methylation status of our entire PCR amplicon including all CpGs covered. Considering that adjacent CpGs are likely to share similar methylation levels [46], we hypothesized that substantial DNA methylation differences could potentially be evident also around the genomic area of our candidate tDMSs and aimed to explore how useful this approach might be for epigenetic MZ twin differentiation. Interestingly, considering all tDMSs analysed so far, we detected inter-twin melting temperature differences in 27 out of the 36 potential twin-differentiating PCR products (75%) in the reference DNA. Two examples are presented in Figure 6. Of course, we cannot exclude the possibility of existing fragment methylation differences between MZ twin pairs in the rest 9 fragments that, nevertheless, cancel each other out at the total

fragment melting temperature. However, out of these 27 PCR products we could only replicate 13 (48.1%) in the corresponding trace DNA, which corresponds to the detected lower DNA methylation differences of our specific CpGs in bloodstains. Taking into account the number of CpGs included in each specific fragment, we calculated that these melting temperature differences translate to 0.01-0.53 fragment methylation differences. However, the advantage of including such melting curve analysis can be seen in top 1st tDMS in twin pair 5 (cg13291296), top 2nd tDMS in twin pair 6 (cg09007841) and top 3rd tDMS in twin pair 9 (cg10617763), that, despite their small methylation differences detected in bloodstains [Figure 5E, 5F and 5H], they show a 0.40, 0.10 and 0.16 methylation difference when the entire fragments are considered. Moreover, the true complementary nature of combining targeted techniques can be seen in four tDMSs that resulted in no substantial differences when only qPCR was employed, but gave substantial fragment methylation differences using high-resolution melting curves (two tDMSs in the 'challenging' MZ twin pair 8: top 1st tDMSs - cg00922825 and top 3rd tDMSs cg10617763). Therefore, we propose that, whenever possible, the entire surrounding region of candidate tDMSs is co-analysed.

(A) Fragment surrounding cg27370028 (**10 CpGs, 154 bp**)



(B) Fragment surrounding cg24371114 (**11 CpGs, 257 bp**)

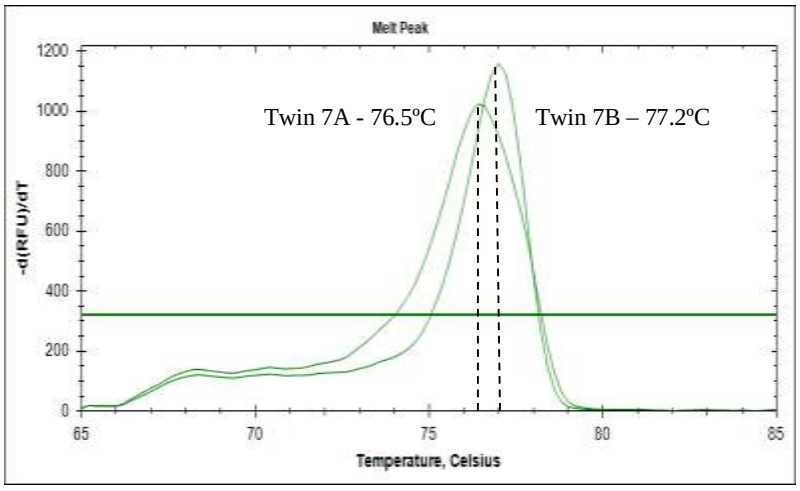


Figure 6. Examples of high-resolution melting curve analysis in MZ twin pair 7 reference DNA for two assays

4. Final discussion and future work

Differentiating between MZ twins for forensic purposes is currently impossible using standard forensic use of DNA via STR profiling. On the other hand, epigenetics and especially DNA methylation profiling has been recently proposed as a suitable approach to overcome this challenge. Hence, the aim of this project was to test the complete scenario of future epigenetic MZ twin differentiation in forensic casework, including the analysis of both reference- and trace-type DNA. During marker discovery, our genome-wide experiment revealed numerous potentially useful candidate tDMSs per MZ twin pair. We understand that on a biological level this number can be influenced by various factors, such as the age and gender of twins, the investigated tissue, potential disease state or phenotype discordance as well as the degree of unique/shared environment between twin individuals within the same pair, and thus, is likely to largely vary between different pairs. Moreover, on a technical level the adopted normalisation strategy can also influence the resulting number of candidate markers, as seen here by using two different methods.

In this study we focused our analysis in blood by testing reference-type whole blood samples and trace-type bloodstains. DNA methylation is known to be tissue-specific [47], therefore it is important to address the limitation of comparing between different tissues by ensuring that both reference and trace samples originate from the same tissue. In case of blood or saliva stains this might be possible, however we understand that in certain cases it might be unlikely that legal authorities will be able to allow for collection of the tissue of interest (such as semen in sexual assault cases). Using a reference sample from a different tissue may not be entirely suitable for comparison to a trace sample from a different tissue origin, unless there are CpG sites that show twin-differentiating DNA methylation across tissues. Nevertheless, this has yet to be determined.

Although numerous candidate tDMSs were obtained from microarray analysis in blood, only a portion (just over two thirds) of the selected candidate markers, and subsequently, another two thirds of the validated markers, resulted in >0.1 difference in reference- and trace-type DNA, respectively. We believe that these findings can be partly explained by technical variation, method-to-method discrepancies and perhaps by the marker selection approach. While differences in the sample analysis (in terms of extraction and bisulfite treatment) between the reference- and trace-type DNA samples can also play a role, DNA from bloodstains seemed to generally provide lower methylation. While bloodstains were collected at the same time point with reference samples, they were subsequently stored at -80^{o}C until qPCR analysis (span of several months), we are uncertain whether this storage could have any significant effect. We suggest that future studies take into account these possibilities of 'losing' potentially informative markers throughout the proposed step-wise analysis.

One factor not considered and tested with our study design, which could lead to further complications in practical forensic epigenetic applications, is the potential in-vivo longitudinal instability of tDMSs identified in reference DNA via genome-wide analysis, as trace and reference samples are typically not collected at the same time point (as done in our study for reasons of simplification). If a tDMS selected from the reference DNA screening analysis changes its methylation status during the time between sampling of reference DNA and placement of trace DNA, this would make such tDMSs non-informative for twin differentiation from trace DNA. In an recent attempt to study the stability of CpG methylation overtime Zhang *et al* [28] analysed blood DNA samples obtained from 10 MZ pairs together with 8 individuals collected at four time points over 9 months using the Illumina Infinium Human Methylation 450K BeadChip array. The authors found a subset of CpG sites (located on CpG islands) with changing methylation over this

short time period, but detected no significant overall epigenetic drift within the same individual [28].

Another aim of our manuscript was to provide a forensically suitable alternative for accurate and reproducible DNA methylation detection that could not only provide single CpG-resolution, but is also cheap and allows for high-throughput. Although we understand that the nested qPCR design we developed might introduce extra variation in DNA methylation detection accuracy, its set-up will eventually allow for the construction of a twin-specific multiplex end-point PCR, which can increase the overall sensitivity and allow for the analysis of very low quantity/quality samples. Initial validation showed that 50 pg of starting DNA material were sufficient for complete and accurate methylation profiles (data not shown). Nevertheless, further validation including extensive reproducibility and repeatability testing is still required to fully assess and explain the variability of detected DNA methylation, as observed between whole blood reference samples and minute bloodstains, which is expected to be tDMS-dependent. One can also consider applying potentially more specific and sensitive probe-based qPCR approaches, such as MethyLight [48], which can also be useful from a forensic standpoint.

Taking into account the observed variation and the potential effect of various technical and biological factors, and as indicated by our data from one example MZ pair, future forensic twin differentiation might require the analysis of a large number of twin-specific markers to allow individual identification of MZ twins from epigenetic data. However, analysing a large number of CpGs (at hundreds or thousands level) via qPCR or other similar targeted approaches might appear not practical. On the other hand, genome-wide technologies, such as microarray analysis or whole genome bisulfite sequencing, are not practical either as they require DNA qualities and quantities typically not available from crime scene materials. Technologies, such as targeted bisulfite

sequencing using massively parallel sequencing platforms expectedly allow for the parallel analysis of more CpGs (at least 16-24 CpG loci, [49, 50]) from low-quality and -quantity DNA compared to what is possible with other targeted approaches, such as qPCR and pyrosequencing. However, future work is still needed to show how many DNA methylation markers can be multiplexed with such technologies. Nevertheless, as far as we see it, the ideal approach for forensic MZ twin differentiation would use the same highly-sensitive and reliable, non-targeted epigenetic screening technology in reference- and trace-type DNA, providing a large number of tDMSs for individual identification of MZ twins, which at this moment is not available.

Future work is also needed to establish from a statistical point of view how many tDMSs are required for trustworthy individual identification of MZ twins from crime scene material. While the purpose of this study was not to propose a valid statistical approach on how to convey the strength of DNA methylation evidence towards reliable MZ identification, we understand that this is an important aspect that has not been demonstrated yet. Future studies are needed to 'translate' methylation differences into probabilities of observing such methylation levels under two hypotheses (e.g. twin A being the stain donor *vs*. twin B being the stain donor), which should consider the best way to combine methylation data from different CpG sites (their methylation of which might not be independent) and how to calculate methylation level frequencies. Blind testing to compare the methylation profile of a bloodstain vs. the methylation profiles of the twins' reference samples is expected to reveal how well assignment to the 'right' twin can be made and how often discrepancies are observed, however this will require an advanced and complex statistical approach.

5. Conflict of interest

The authors declare no conflict of interest.

6. Acknowledgments

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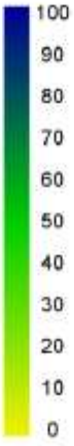
Twin pair	Twin	Array-derived candidate tDMSs (DNA methylation ratio)		
		top 1§	top 2§	top 3§
1	**A**	0.558	0.158	0.647
	B	0.682	0.298	0.368
	Diff*	**0.124**	**0.140**	**0.279**
2	**A**	0.609	0.365	0.446
	B	0.609	0.111	0.249
	Diff	**0.000**	**0.254**	**0.197**
3	**A**	0.856	0.430	0.000
	B	0.766	0.846	0.705
	Diff	**0.090**	**0.416**	**0.705**
4	**A**	0.000	0.342	0.122
	B	0.586	0.891	0.598
	Diff	**0.586**	**0.549**	**0.476**
5	**A**	1.000	0.152	0.066
	B	0.715	0.867	0.323
	Diff	**0.285**	**0.715**	**0.257**
6	**A**	0.407	0.762	*N/A*
	B	0.770	0.566	
	Diff	**0.363**	**0.196**	
7	**A**	0.982	0.073	0.460
	B	0.612	0.080	0.527
	Diff	**0.370**	**0.007**	**0.067**
8	**A**	0.199	1.000	0.833
	B	0.190	1.000	0.882
	Diff	**0.009**	**0.000**	**0.049**
9	**A**	0.689	0.451	0.685
	B	1.000	0.781	0.933
	Diff	**0.311**	**0.330**	**0.248**
10	**A**	*N/A*	0.403	0.075
	B		0.385	0.071
	Diff		**0.018**	**0.004**

*****Within-pair absolute DNA methylation difference between twin A and twin B - Expected >30%



DNA methylation levels

Table 1. Summary of qPCR results of array-derived candidate tDMSs in the same reference-type DNA used for array analysis for the three top markers per MZ twin pair